Field Efficacy and Seasonal Expression Profiles for Terminal Leaves of Single and Double *Bacillus thuringiensis* Toxin Cotton Genotypes

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ABSTRACT Evaluation of commercial Cry1Ac transgenic *Bacillus thuringiensis* Berliner (Bt) cotton varieties (Bollgard, Monsanto, St. Louis, MO) and an experimental Cry1Ac + Cry2Ab transgenic Bt cotton variety (Bollgard II, Monsanto) for lepidopteran field efficacy was conducted during the 2000 growing season. In addition, a commercially available (Envirologix, Portland, ME) quantification assay (ELISA) was used to measure and profile the expression levels of Cry proteins in two of these varieties ['DP 50B, Bollgard'; 'DP 50BII, Bollgard II' (Delta & Pine Land, Scott, MS)]. Populations of beet armyworms, *Spodoptera exigua* (Hübner), and soybean loopers, *Pseudoplusia includens* (Walker), were significantly lower ($P < 0.05$) in Bollgard II plots compared with Bollgard. Population numbers for fall armyworms, *Spodoptera frugiperda* (J. E. Smith), and salt marsh caterpillars, *Estigmene acrea* (Drury), were lower in Bollgard II plots compared with Bollgard but means did not differ significantly ($P > 0.05$). Single and dual-toxin genotypes remained superior ($P < 0.05$) compared with conventional cotton against the tobacco budworm, *Heliothis virescens* (F.). The addition of Cry2Ab had no significant ($P > 0.05$) impact on Cry1Ac expression in Bollgard II compared with Cry1Ac expression in Bollgard. Furthermore, throughout the season Cry2Ab was present at much higher levels in the plant compared with Cry1Ac for Bollgard II plants. Possible species-specific reasons for increased efficacy of Bollgard II over Bollgard are discussed.

KEY WORDS transgenic cotton varieties, ELISA, *Bacilllus thuringiensis* quantification, dual-toxin

SINCE TRANSGENIC CRY1AC *Bacillus thuringiensis* Berliner (Bt) cotton (Bollgard, Monsanto, St. Louis, MO) became widely commercialized in the United States in 1996, growers and researchers have noted that populations of several lepidopteran pests that survive in these genotypes are not controlled with this technology alone (Bacheler and Mott 1997; Smith 1997, 1998). Although this technology is highly effective against the tobacco budworm, *Heliothis virescens* (F.), and the pink bollworm, *Pectinophora gossypiella* (Saunders) (Williams 2000), supplemental foliar insecticide applications (e.g., pyrethroids) have been used in many Bollgard fields to control economically damaging populations of fall armyworms, *Spodoptera frugiperda* (J. E. Smith), and especially bollworms, *Helicoverpa zea* (Boddie), (Bacheler and Mott 1997, Roof and Durant 1997, Smith 1998, Burd et al. 1999). Laboratory studies have shown that the addition of a second Bt protein (Cry2Ab) to Bollgard (Bollgard II, Monsanto) may reduce the survival of Lepidoptera that are often found in Bollgard cotton (Greenplate et al. 2000b, Stewart et al. 2001). However, few field studies to date have been conducted to evaluate the efficacy of a dual-protein transgenic cotton genotype against a multitude of lepidopteran pests (Stewart and Knighten 2000).

Differences in Cry1Ac expression levels in leaves among Bollgard varieties have been correlated to survival differences in various Lepidoptera that are intrinsically tolerant to Bt both in the laboratory and in the field (Adamczyk et al. 2001a, Layton 2000). Profiling season-long expression of Cry1Ac in Bollgard varieties has shown that the δ-endotoxin level decreases as the plant ages (Fitt 1998, Greenplate et al. 2000a; Adamczyk et al. 2001a, 2001b). In addition, season-long expression differences among Cry1Ac varieties can vary as much as two-fold throughout the season (Adamczyk et al. 2001b) while plant structures, such as terminal leaves, express more δ-endotoxin compared with others, such as flowers (Greenplate 1999, Greenplate et al. 2000a, Adamczyk et al. 2001a). Reasons for the decreased and differential expression are not fully understood but appear to be mRNA related (Finnegan et al. 1998). The addition of Cry2Ab to Bollgard cotton may provide increased efficacy against Cry1Ac intrinsically tolerant Lepidoptera by increasing the dose and/or toxicity to the insect. The purpose of this research was to evaluate the efficacy of an experimental transgenic cotton (Bollgard II) against various lepidopteran pests of cotton as well as to profile season-long expression of both proteins.

Materials and Methods

Field Plots. Three varieties (conventional, 'DP 50'; transgenic Cry1Ac Bollgard, 'DP 50B'; and transgenic

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Table 1. Mean ± SEM number of Lepidoptera found in Bollgard and Bollgard II plots using a 1.24-m^2 drop cloth

Variety	Artificial infestations[a]		Natural infestations[b]			
	BAW	FAW	BAW	TBW	SBL	SMARSH
DP 50[c]	42.00a ± 9.35	3.00a ± 0.91	21.00a ± 6.34	7.00a ± 3.24	32.00a ± 5.67	8.75a ± 6.55
DP 50B[d]	35.50a ± 7.68	1.75a ± 0.75	8.75a ± 5.06	0.25b ± 0.25	37.75a ± 7.41	2.75a ± 1.11
DP 50BII[e]	2.50b ± 0.64	0.75a ± 0.75	6.00a ± 6.00	0b	0.25b ± 0.25	0.25a ± 0.25
F-value	43.03	4.39	3.51	9.92	106.77	2.06
df	2, 6	2, 6	2, 6	2, 9	2, 9	2, 6
$P > F$	<0.001	0.067	0.098	0.005	<0.001	0.208

Number of insects was log transformed prior to analysis. Means in column followed by the same letter are not significantly different (α = 0.05; PROC MIXED [Littell et al. 1996]). BAW, beet armyworm; FAW, fall armyworm; TBW, tobacco budworm; SBL, soybean looper; SMARSH, salt marsh caterpillar.

[a] Larval population attributed to inoculations with laboratory colonies was rated on 26 July 2001.

[b] Naturally occurring larval populations was rated on 23 August 2001.

[c] Conventional near-isogenic line ('DP' = Delta & Pineland variety, Delta & Pineland, Scott, MS).

[d] Contains Cry1Ac endotoxin (Bollgard, Monsanto, St. Louis, MO).

[e] Contains Cry1Ac and Cry2Ab endotoxins (Bollgard II, Monsanto).

Cry1Ac + Cry2Ab Bollgard II, 'DP 50 BII') were planted in experimental plots on 23 May 2000 near Elizabeth, MS, under a 1-yr Experimental Use Permit (EUP) (USDA Reference # 00-062-02N). Plots consisted of four rows (1.0-m centers) by 9.15 m treatments arranged in a randomized complete block design with each variety replicated four times (once in each block). Only insecticides not active on Lepidoptera were applied to all plots throughout the season as dictated by local management practices. Plots were irrigated twice.

Insects. To ensure that data would be generated for occasional lepidopteran pests of cotton, a colony of fall armyworms and a colony of beet armyworms, *Spodoptera exigua* (Hübner), were established. Larvae (≈300) of fall armyworm were collected from whorl-stage field corn near Stoneville, MS, in April 2000. To ensure genetic diversity and traits present in field individuals, ≈500 adult male beet armyworms were collected using pheromone traps located near Nepanee, MS, in December 1999 and mated with a laboratory colony of females to establish a new colony. Both species were reared for one complete generation in the laboratory as described by Adamczyk et al. (1998), and the subsequent generation used for field inoculations.

Inoculations of beet armyworm and fall armyworm egg masses to plants for all varieties were conducted in late July 2000. In the laboratory, egg masses of either species were deposited on nylon cloth placed on the top of adult rearing cages (3.79-liter cardboard containers). For each inoculation, an egg mass of equal size (≈200–300 eggs/2.54-cm^2 cloth sample) was stapled to the underside of a mature leaf in all plots. For fall armyworms, each plot received 24 egg masses over a two-week period. For beet armyworms, each plot received 10 egg masses on 17 July 2000.

Natural infestations of many lepidopteran species [beet armyworm; tobacco budworm; soybean looper, *Pseudoplusia includens* (Walker); salt marsh caterpillar, *Estigmene acrea* (Drury)] occurred in all plots. Lepidoptera were sampled (three subsamples/plot) using a 1.24-m^2 drop cloth. Collections of heliothine larvae from all plots were identified to be mainly tobacco budworms (>95%). In addition, because fall armyworms are commonly found low in the plant canopy and associated with white and pink flowers (Luttrell and Mink 1999), scouting of entire plants (10/plot) and flowers (25/plot) was conducted. During peak flowering, damage to squares (flower buds) significant to cause square abscission (from 10 entire plants/plot) were recorded. Likewise, damage to pink flowers (25/plot) caused by lepidopteran feeding on petals also were recorded. Furthermore, the number of bolls/plant (20/plot), and subsequently the proportion of damaged bolls [i.e., larval penetration significant to cause fruit loss as described in Adamczyk et al. (1997)] were recorded one week before the opening of bolls.

***Bacillus thuringiensis* Quantification.** Commercially available Bt quantification kits (#AP003 and #AP005, Envirologix, Portland, ME) were used to profile season-long expression of Cry proteins in Bollgard and Bollgard II varieties. Because these test kits have been designed primarily to quantify Cry endotoxin levels in leaf tissue, this plant structure was evaluated throughout the season. For each sample date (8), a single terminal leaf (first uncurled, main stem) was randomly harvested from 10 plants/plot for all varieties (four replications/variety). Leaves were transported to the laboratory and within a few hours after being harvested, one sample (≈5–8 mg) was taken from each leaf using a standard 6-mm paper ticket punch. The leaf samples were weighed to accurately determine the amount of starting material and combined (i.e., pooled) for each variety/plot into a 1.5-ml microcentrifuge tube containing extraction buffer. The tissue was then homogenized using a mini-beadbeater-8 (Biospec Products, Bartlesville, OK) using 6.4-mm steel ball bearings.

To quantify the amount of Bt present for each variety (Cry1Ac in DP 50B or Cry1Ac and Cry2Ab in DP 50BII), a commercial quantification plate kit was used as described in Adamczyk et al. (2001a). It should be noted that the Cry2A test kit (#AP005) contains Cry2Aa2 calibrators, and the cross-reactivity between Cry2Aa2 and Cry2Ab antibodies is only ≈12–15%. Therefore, it is likely that the reported Cry2Aa2 values

(ppm) for DP 50BII is much lower than true Cry2Ab values. Consequently, for all sample dates, varieties were always identically compared in a side-by-side experiment (i.e., the same sample homogenates were tested with antibodies at the same time) to ensure relative comparisons. The proper standard curve, dilution factors, positive and negative controls, calibrators, and calculations were conducted as dictated in the kit protocols.

Statistics. All means for the number of Lepidoptera species, bolls, and damaged fruiting structures found in plots were log-transformed and analyzed using REML—analysis of variance (ANOVA). The means were then separated using the LSMEANS option of PROC MIXED (Littell et al. 1996). In addition, means for expression of Cry1Ac in Bollgard varieties were analyzed using the Repeated Measures statement (compound symmetry) of PROC MIXED (Littell et al. 1996).

Results and Discussion

Fewer lepidopterous pests were found in Bollgard II plots (DP 50BII) compared with Bollgard (DP 50B) or conventional cotton (DP 50). The addition of Cry2Ab to transgenic cotton containing Cry1Ac significantly increased efficacy against beet armyworm and soybean looper. Salt marsh caterpillars and fall armyworms were found in lower numbers but means did not differ significantly. Activity of both single and double toxin genotypes remained superior compared with conventional cotton against tobacco budworm (Table 1). Bollgard II was the only transgenic cotton technology that significantly reduced fall armyworm numbers in pink flowers and throughout the entire plant compared with conventional cotton (Table 2). Because fall armyworms are primarily associated with flower damage and subsequent boll damage in Bollgard cotton (Smith 1997, 1998), Bollgard II may increase efficacy against Lepidoptera that primarily feed on reproductive structures (Table 3).

The lepidopteran bioactivity and protein expression profiles of Cry2Ab appear to be quite different than Cry1Ac. Sims (1997) noted that soybean loopers were highly sensitive (LC_{50} = 0.06 μg/ml) to purified Cry2A (no reference to subclass) incorporated into artificial diet, whereas other studies have shown that they are not as sensitive to Cry1Ac in Bollgard (Sumerford and Solomon 2000). However, Greenplate et al. (2000b) stated that Cry2Ab is less potent than Cry1Ac against tobacco budworms on an equal dose basis. The addition of Cry2Ab to Cry1Ac transgenic cotton could increase lepidopteran activity because of increased overall expression, especially in the reproductive tissues, of Cry2Ab over Cry1Ac (10-fold) (Greenplate et al. 2000b). Although not always significantly different from Bollgard, Bollgard II had very few damaged reproductive structures (Table 3; Fig. 1). In our study involving terminal leaves, we also observed that Cry2Ab is expressed at a higher level than Cry1Ac throughout the growing season (Fig. 2); however, we did not profile expression in the fruiting structures. Thus, depending on the species, the increased activity of Bollgard II compared with Bollgard can be due to increased potency of Cry2Ab, increased overall expression level of Cry2Ab, or possibly a synergistic combination.

Achieving season-long dual-toxin activity in Bollgard II will be a critical element in resistance man-

Table 2. Mean ± SEM number of fall armyworms found in Bollgard and Bollgard II flowers and entire plants

Variety	25 flowers/Plot		
	White flowers	Pink flowers	10 entire plants/Plot
DP 50[a]	1.50a ± 0.96	2.25a ± 0.95	2.75a ± 0.75
DP 50B[a]	0.25a ± 0.25	0.75ab ± 0.48	2.50ab ± 1.19
DP 50BII[a]	0.25a ± 0.25	0b	0.25b ± 0.25
F-value	1.16	6.14	4.25
df	2, 6	2, 9	2, 9
$P > F$	0.375	0.021	0.050

Number of insects was log transformed prior to analysis. Means in column followed by the same letter are not significantly different (α = 0.05; PROC MIXED [Littell et al. 1996]). The fall armyworm population was attributed to inoculations with a laboratory colony (see *Materials and Methods*).

[a] See footnotes to Table 1.

Table 3. Mean ± SEM number of damaged squares and flowers in Bollgard and Bollgard II plots

Variety	Squares[b]	Pink Flowers[c]
DP 50[a]	7.75a ± 3.07	7.75a ± 1.49
DP 50B[a]	6.25a ± 2.17	6.50a ± 1.04
DP 50BII[a]	0.75b ± 0.48	3.50b ± 0.65
F-value	6.10	5.18
df	2, 6	2, 9
$P > F$	0.036	0.032

Number of damaged reproductive structures was log transformed prior to analysis. Means in column followed by the same letter are not significantly different (α = 0.05; PROC MIXED [Littell et al. 1996]).

[a] See footnotes to Table 1.

[b] Damage significant to cause fruit abscission. Ten entire plants examined/plot.

[c] Lepidopterous feeding on bracts or petals. 25 flowers examined/plot.

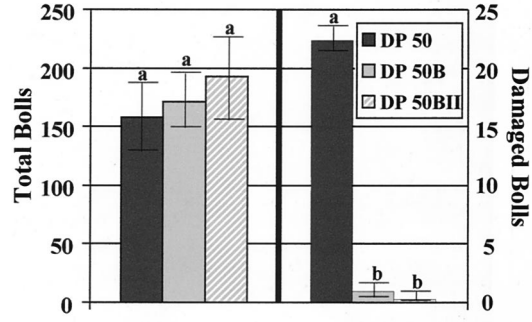

Fig. 1. Total and damaged number of bolls from 20 examined plants/plot of conventional (DP 50), Bollgard (DP 50B), and Bollgard II (DP 50BII) cotton. Means were log-transformed before analysis. Column bars containing the same letter are not significantly different (α = 0.05); PROC MIXED (Littell et al. 1996).

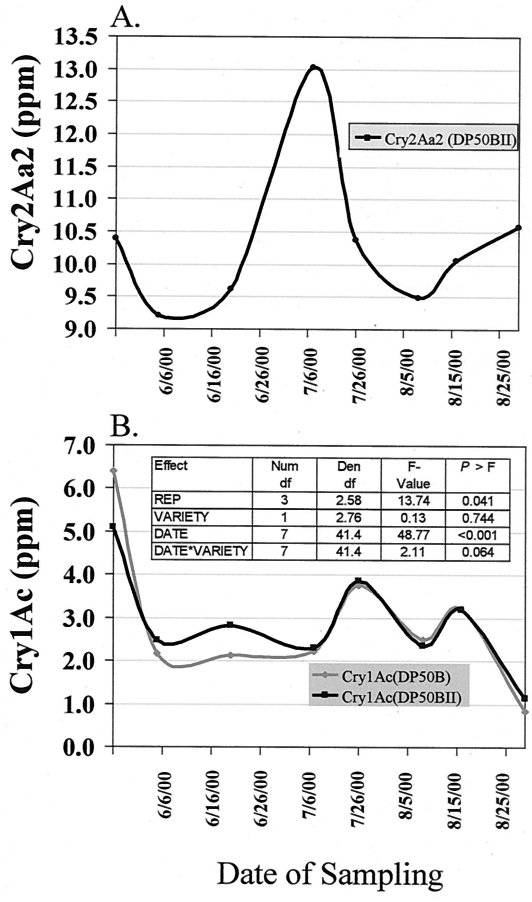

Fig. 2. Amount of Cry1Ac and Cry2Ab endotoxin (ppm) measured throughout the season in terminal leaves from (A). Bollgard II (DP 50BII) and (B). Bollgard (DP 50B) plots. Because Cry2Aa2 antibodies were used to quantify Cry2Ab in DP 50BII, reported ppm values are most likely low (see *Materials and Methods*). Means for expression of Cry1Ac in Bollgard varieties were analyzed using the Repeated Measures statement (compound symmetry) of PROC MIXED (Littell et al. 1996).

agement strategies for tobacco budworms and bollworms. It is imperative that both toxins provide additive activity with minimal cross-resistance to slow the development of resistance (Tabashnik 1994, Gould 1998). Sachs et al. (1998) noted that the expression profiles for subclasses of the Cry1A protein (c and b) were very different when transformed into the same cotton line. Not surprisingly, expression profiles of the two Cry proteins in Bollgard II appeared to be different from one another (Fig. 2). However, our data further indicated that the addition of Cry2Ab had no deleterious effect on levels of Cry1Ac in Bollgard II (also suggested by Greenplate et al. 2000b) and that both proteins were present throughout the season (see Fig. 2 insert). The cultivar DP 50BII results from cloned plants regenerated from transformed tissue of DP 50B (Bollgard) and backcrosses with elite varieties will have to be conducted before commercialization (Greenplate et al. 2000b). Because current Bollgard varieties differ in the amount of expressed Cry1Ac (Adamczyk et al. 2001a, 2001b), future research must be conducted with commercial varieties of Bollgard II to ensure that expression levels of both proteins are adequate to provide season-long dual-protein activity against target Lepidoptera.

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